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(54) Title: MODULATION OF ENZYME ACTIVITIES IN THE *IN VIVO* CLONING OF DNA

(57) Abstract

    Methods and recombinant vectors suitable for accomplishing the *in vivo* alteration of a nucleic acid molecule are disclosed. The invention in particular discloses the use of recombinases such as Cre to accomplish *in vivo* recombination.

TITLE OF THE INVENTION:

MODULATION OF ENZYME ACTIVITIES IN THE
IN VIVO CLONING OF DNA

FIELD OF THE INVENTION:

5   The invention relates to vectors and methods for
modulating the expression of enzyme activities, such as
ligases, nucleases, and recombinases, in order to thereby
facilitate the in vivo cloning of DNA molecules.

CROSS-REFERENCE TO RELATED APPLICATIONS:

10   This application is a continuation-in-part of U.S.
Patent Application Serial No. 07/825,267 (filed January
24, 1992).

BACKGROUND OF THE INVENTION:

        The techniques of molecular biology have found
15   extensive use in the cloning and analysis of DNA
molecules.   The most commonly used methods for cloning a
gene sequence involve the in vitro use of site-specific
restriction endonucleases, and ligases.   In brief, these
methods rely upon the capacity of the "restriction
20   endonucleases" to cleave double-stranded DNA in a manner
that produces termini whose structure (i.e. 3' overhang,
5' verhang, or blunt nd) and sequenc are both well
defin d.   Any such DNA molecule can then b j in d to a
suitably cleav d vector molecule (i.e. a nucleic acid

-2-

molecule, typically double-stranded DNA, having
specialized sequences which permit it to be replicated in
a suitable host cell) through the action of a DNA ligase.
The gene sequence may then be duplicated indefinitely by
5   propagating the vector in a suitable host. Methods for
performing such manipulations are well-known (see, for
example, Perbal, B. A Practical Guide to Molecular
Cloning, John Wiley & Sons, NY, (1984), pp. 208-216;
Maniatis, T., et al. (In: Molecular Cloning, A Laboratory
10   Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
(1982); Old, R.W. et al., In: Principles of Gene
Manipulation, 2nd. Ed., University of California Press,
Los Angeles, (1981), all herein incorporated by
reference).

15   Depending upon the size and characteristics of the
desired target molecule, any one of the three different
types of vectors -- plasmids, bacteriophage, or cosmids --
can be employed (See, generally, Watson, J.D., In:
Molecular Biology of the Gene, 4th Ed., W.A. Benjamin,
20   Inc., Menlo Park, CA (1987), which reference is
incorporated herein by reference).

          The use of plasmids in cloning is quite well known
(see, for example, Cohen et al., U.S. Patent 4,237,224;
Itakura, U.S. Patent 4,356,270; Fraley, R.T. et al., PCT
25   Application WO 84/02919; etc.). In general, the most
pronounced deficiency of plasmid vectors is the relatively
small amount (up to about 5-10 kb) of DNA which can be
cloned into them. In general, the larger the size of the
target molecule, the lower the efficiency of plasmid
30   transformation.

          Bacteriophage vectors, and particularly vectors
engineered from the bacteriophage λ have been extensively
used as cloning vehicles (see generally, The Bacteriophage
Lambda, (Hershey, A.D., ed.), Cold Spring Harbor Press,
35   Cold Spring Harbor, NY (1971), and Lambda II, (Hendrix, R.
et al., Eds.), Cold Spring Harbor Press, Cold Spring
Harbor, NY, pp. 175-209 (1983), Maniatis, T., et al. (In:

Molecular Cloning. A Laboratory Manual, Cold Spring Harbor
Press, Cold Spring Harbor, NY (1982), all herein
incorporated by reference). In general, bacteriophage
vectors have the advantage that they can be used to clone
gene sequences of up to approximately 23 kb.

Cosmids are vectors which have been specifically
designed to facilitate the cloning of large DNA molecules.
The essential components of a cosmid vector are (1) a
drug-resistance marker; (2) a plasmid origin of
replication; (3) one or more unique cloning sites that are
recognized by a restriction endonuclease; (4) a _cos_ site
of bacteriophage λ. Cosmid cloning methods are described,
for example, by Maniatis, T., _et al._ (In: _Molecular
Cloning. A Laboratory Manual_, Cold Spring Harbor Press,
Cold Spring Harbor, NY (1982)). Cosmids vectors can be
used to clone 40-50 kb of target molecule. They are thus
highly useful in the cloning and analysis of eukaryotic
DNA, and especially mammalian genomes. Despite the ease
with which _in vitro_ cloning manipulations are often
accomplished, serious impediments often limit their
application in specific instances.

In vitro cloning may result in genetic
rearrangements, deletions or insertions occurring in the
desired gene sequence. It often requires the substantial
prior purification of gene fragments. Such purification
may be impeded if the desired gene fragment does not
amplify at the same rate, or with the same fidelity, as
other fragments. These concerns are particularly
important with regard to the cloning of large DNA
fragments, such as those containing human genes and gene
families.

Moreover, the capacity to move target molecules from
one vector to another _in vitro_ is often limited by the
availability or suitability of r striction sites. For
example, in order to complete a desired cl ning
manipulation, it may be necessary to remove a particular
enzym that was needed in an initial cloning st p. Such

-4-

removal would necessitate a time-consuming purification of
the cloning intermediate. Thus, a method for effecting
the transfer of a target molecule from one vector to
another that would not require such intermediate
5   processing would be desirable. The present invention
provides such a method and vector molecules for use
therein.

SUMMARY OF THE INVENTION:

The invention relates to a method for the _in vivo_
10   manipulation of a cloned target molecule. The method uses
a vector molecule that is capable of expressing an
activity that acts on a different nucleic acid molecule in
order to facilitate the cloning or manipulation of the
target molecule.
15   In detail, the invention provides a method for
accomplishing _in vivo_ alteration of a target molecule in
a host cell which comprises the steps:
a)   providing to the host cell a first vector, the
vector containing (i) a preselected gene and (ii) a
20   replicon, wherein the preselected gene is capable of being
expressed in the host cell, and the replicon is sufficient
to permit the replication of the vector;
b)   providing to the host cell a second vector, the
vector containing (i) the target molecule which is to be
25   altered, (ii) a replicon, and (iii) a determinant that has
the capacity to inhibit the expression of the preselected
gene of the first vector;
c)   culturing the host cell under conditions
sufficient to permit the expression of the preselected
30   gene, to thereby mediate the desired alteration of the
target molecule; wherein the second vector inhibits the
expr ssion, and th reby results in the modulati n of the
expression of th pr s l cted gene.
The invention als includ s a h st cell containing a
35   first and a second vect r, wher in the first vector

contains (i) a preselected gene and (ii) a replicon,
wherein the preselected gene is capable of being expressed
in the host cell, and the replicon is sufficient to permit
the replication of the vector; and

5   wherein the second vector contains (i) a target molecule,
and (ii) a replicon, and a determinant that has the
capacity to inhibit the expression of the preselected gene
of the first vector.

The invention also provides a kit, being specially

10   adapted to contain in close compartmentalization:

    a)   a first container which contains a first vector
which contains a replicon and a preselected gene, the
vector being capable of expressing the preselected gene in
a host cell;

15   b)   a second container, the container containing a
second vector, the vector having a determinant that has
the capacity to inhibit the expression of the preselected
gene of the first vector, and being specially adapted to
facilitate the introduction of a desired target molecule.

20   In particular, the invention includes the embodiments
of the above method and host cell and kit wherein the
determinant of the second vector inhibits the expression
of the preselected gene of the first vector by expressing
a gene present on the second vector, wherein the expressed

25   gene of the second vector effects the excision of the
preselected gene from the first vector, and wherein the
determinant of the second vector inhibits the expression
of the preselected gene of the first vector by comprising
an incompatibility determinant sufficient to inhibit the

30   replication of the first vector, and thereby causing the
loss of the vector from the host cell.

The invention particularly includes the embodiment
wherein the alteration is a recombinational alteration,
and wh rein th preselected gene is a rec mbinase gene

35   (especially the cre gene), and the target m lecule
possesses at least one site recognized by the r combinase
(such as loxP).

-6-

DESCRIPTION OF THE PREFERRED EMBODIMENTS:

The present invention provides a method for achieving
the _in vivo_ cloning or manipulation of a target nucleic
acid molecule. In a preferred embodiment, the method
5   employs a vector, typically a plasmid, that contains, and
is capable of expressing, a preselected gene whose
expression product has an enzymatic activity that is
desired in order to effect the cloning or manipulation of
the target molecule. The vector is present in a cell,
10   designated herein as the "host" cell. Any prokaryotic or
eukaryotic cell may be used as the host cell of the
present invention. The preferred prokaryotic host cell is
E. coli. Yeast and mammalian cells comprise examples of
preferred eukaryotic host cells.
15   The target molecule can be introduced into the host
cell by electroporation, transfection, transformation,
fusion, or any other suitable means. Once present within
the host cell, the target sequence is acted upon by the
enzymatic activity that results from the expression of the
20   preselected gene. The vector (or other molecule) that
contains the preselected gene is preferably resident in
the host cell prior to the introduction of the target
molecule. However, this is not a requirement of the
invention, and the nucleic acid molecules that contain the
25   target molecule and the preselected gene can be introduced
simultaneously, or in any order.

I.   The Preselected Gene

The present invention employs a preselected gene that
is capable of being expressed in the host cell. The gene
30   is preferably present in a vector, most preferably a
plasmid vector, however, it may be introduced as a non-
replicating nucleic acid fragment (DNA or RNA) (i.e. it
need not be a vector).

-7-

The expression of the preselected gene will result in the production of a protein that has the ability to interact with the target molecule to thereby alter the structure or characteristic of the target. For example, the preselected gene may encode a ligase, a restriction endonuclease (such as NotI, NdeI or NcoI), a recombinase, a reverse transcriptase, a methylase, etc. The nucleic acid molecule that contains the preselected gene may be constructed such that it contains and expresses only one such preselected gene, or any combination of such genes (such as two or more different methylases, a recombinase and a reverse transcriptase, etc.).

When the preselected gene is a ligase gene, the present invention can be used to mediate the ligation of linear target sequences into a linearized vector. Such linearized molecules may be introduced into the host cell via transformation, electroporation, etc. Thus, such preselected genes are desirable in facilitating the cloning of target fragments.

When the preselected gene is a restriction enzyme gene, the present invention can be used to mediate the site-specific, _in vivo_ cleavage of the target molecule. Thus, such preselected genes are desirable in facilitating the purification or sub-cloning of target fragments.

When the preselected gene is a methylase gene, the present invention can mediate the site-specific, _in vivo_ modification of the target molecule so as to render it resistant to restriction endonuclease cleavage. Thus, such preselected genes are desirable in facilitating the cloning of intact target molecules.

When the preselected gene is a reverse transcriptase gene, the present invention can be used to accomplish the cDNA cloning of any mRNA species present in the host cell.

In a preferred embodiment the preselected gene will be a recombinase g ne. The product express d by such a gene -- a "recombinase" -- is an nzymatic activity that is capable of recombining two nucl ic acid m lecules.

-8-

Thus, such preselected genes can be used to mediate the transfer of a linear target molecule into a vector molecule, or the transfer of the target molecule from one vector to another. Significantly, such transfer is not dependent upon the presence of restriction sites at the termini of the target sequence. Likewise, such transfer is not precluded by the presence of restriction sites within the target sequence. All that is required is that the target sequence be flanked by sites recognized by the recombinase. Where the target molecule is a linear molecule, such sites may be naturally present at the termini, but more commonly will be added to the target sequence via ligation or primer extension. Where the target sequence is a circular molecule, such sites can be introduced into molecule via restriction enzymes, homologous recombination, or site-specific recombination.

Two classes of recombinases are known. A "general recombinase" is an enzymatic activity that is capable of participating in a process which results in the scission of two nucleic acid molecules having identical or substantially similar sequences (i.e. "homologous"), and the ligation of the two molecules such that one region of each initially present molecule becomes ligated to a region of the other initially present molecule (Sedivy, J.M., Bio-Technol. 6:1192-1196 (1988), which reference is incorporated herein by reference). The most characterized general recombination system is that of the bacteria E. coli (Watson, J.D., In: Molecular Biology of the Gene, 4th Ed., W.A. Benjamin, Inc., Menlo Park, CA (1987); Smith, G.R., In: Lambda II, (Hendrix, R. et al., Eds.), Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 175-209 (1983)). The E. coli system involves the protein, RecA, which in the presence of ATP or another energy source, can catalyze the pairing of DNA molecul s at regions of hom logy.

In the most preferred embodiment of the present invention, the pr selected gen will ncod a "site-

specific recombinase." In contrast to a general
recombinase, "site-specific recombinase" can recognize
only certain defined sequences as substrates for
recombination. The more preferred of the site-specific

5   recombinases are the Int, Int/Xis, and Cre recombinases.
The Cre recombinase of bacteriophage P1 is the most
preferred site-specific recombinase. The Cre recombinase
of P1 is discussed by Sternberg, N. _et al._ (_J. Molec._
Biol. _187_:197-212 (1986)), herein incorporated by

10   reference.

The term "Cre" recombinase, as used herein, refers to
a protein having an activity that is substantially similar
to the site-specific recombinase activity of the Cre
protein of bacteriophage P1 (Hamilton, D.L., _et al._, _J._

15   _Mol. Biol._ _178_:481-486 (1984), herein incorporated by
reference). The Cre protein of bacteriophage P1 mediates
site-specific recombination between specialized sequences,
known as "_loxP_" sequences:
(SEQ ID NO:1) 5' ATAACTTCGTATAATGTATGCTATACGAAGTTAT 3'

20   (SEQ ID NO:2) 5' ATAACTTCGTATAGCATACATTATACGAAGTTAT 3'
The _loxP_ site has been shown to consist of a double-
stranded 34 bp sequence (SEQ ID NOS: 1 and 2). This
sequence contains two 13 bp inverted repeat sequences
which are separated from one another by an 8 bp spacer

25   region (Hoess, R., _et al._, _Proc. Natl. Acad. Sci. (U.S.A.)_
_79_:3398-3402 (1982); Sauer, B.L., U.S. Patent No.
4,959,317, herein incorporated by reference).

Cre has been purified to homogeneity, and its
reaction with the _loxP_ site has been extensively

30   characterized (Abremski, K., _et al._, _J. Mol. Biol._
_259_:1509-1514 (1984), herein incorporated by reference).
Cre protein has a molecular weight of 35,000. Cre protein
can be obtained commercially from New England
Nuclear/Du Pont. The _cre_ gene (which encodes the Cre

35   prot in) has been cl ned and expressed (Abremski, K., _et_
_al._, _Cell_ _32_:1301-1311 (1983), herein inc rporated by
r ference).

-10-

The Cre protein mediates recombination between two loxP sequences (Sternberg, N., et al., Cold Spring Harbor Symp. Quant. Biol. 45:297-309 (1981)). These sequences may be present on the same DNA molecule, or they may be present on different molecules. Because the internal spacer sequence of the loxP site is asymmetrical, two loxP sites can exhibit directionality relative to one another (Hoess, R.H., et al., Proc. Natl. Acad. Sci. (U.S.A.) 81:1026-1029 (1984)). Thus, when two sites on the same DNA molecule are in a directly repeated orientation, Cre will excise the DNA between the sites (Abremski, K., et al., Cell 32:1301-1311 (1983)). However, if the sites are inverted with respect to each other, the DNA between them is not excised after recombination but is simply inverted. Thus, a circular DNA molecule having two loxP sites in direct orientation will recombine to produce two smaller circles, whereas circular molecules having two loxP sites in an inverted orientation simply invert the DNA sequences flanked by the loxP sites.

Two circular molecules each having a single loxP site will recombine to form a mixture of monomer, dimer, trimer, etc. circles. Higher concentrations of circles favor higher n-mers; lower concentrations of circles favor monomers.

A circular DNA molecule having a single loxP site will recombine with a linear loxP-containing DNA molecule to produce a larger linear molecule. The converse reaction of a linear molecule with direct repeats of loxP sites is thus the production of a circle containing the sequences between the loxP sites, and remaining linear. However, if the loxP sites are inverted repeats, recombination flips the sequence between the loxP sites back and forth. Thus, the interaction of loxP and Cre can cause either circularization of a linear molecule or the linearization of an appropriate circularized molecule.

In one embodiment, the Cre protein may be the expression product of a mutated cre gene, and may interact

-11-

with mutated loxP sites. Suitable mutations have been
produced both in Cre, and in the loxP site. The Cre
mutants thus far identified have been found to catalyze
recombination at a much slower rate than that of the wild-
type Cre protein. loxP mutants (such as LoxP511) have
been identified which recombine at lower efficiency than
the wild-type site (Abremski, K., et al., J. Biol. Chem.
261:391-396 (1986); Abremski, K., et al., J. Mol. Biol.
202:59-66 (1988), herein incorporated by reference).

It has been found that certain E. coli enzymes
inhibit efficient circularization of linear molecules
which contain two loxP sites. Hence, enhanced
circularization efficiency can be obtained through the use
of E. coli mutants which lack exonuclease V activity
(Sauer, B., et al., Gene 70:331-341 (1988)).

Cre has been able to mediate loxP specific
recombination in Saccharomyces cerevisiae (Sauer, B.,
Molec. Cell. Biol. 7:2087-2096 (1987); Sauer. B.L., U.S.
Patent No. 4,959,317, herein incorporated by reference),
and in mammalian cells (Sauer, B., et al., Proc. Natl.
Acad. Sci. (U.S.A.) 85:5166-5170 (1988), Sauer, B., et
al., Nucleic Acids Res. 17:147-161 (1989), both references
herein incorporated by reference). Similarly, the
recombination system has been capable of catalyzing
recombination in plant cells (Dale, E.C., et al., Gene
91:79-85 (1990)).

A large number of alternative suitable site-specific
recombinases have been described, and their genes can be
used in accordance with the method of the present
invention. Such recombinases include the Int recombinase
of bacteriophage λ (with or without Xis) (Weisberg, R. et
al., In: Lambda II, (Hendrix, R. et al., Eds.), Cold
Spring Harbor Press, Cold Spring Harbor, NY, pp. 211-250
(1983), herein incorporat d by reference), TpnI and the
β-lactamase transposons (Levesqu , R.C., J. Bacteriol.
172:3745-3757 (1990)); th Tn3 resolvas (Flanagan, P.M.
et al., J. Molec. Biol. 206:295-304 (1989); Stark, W.M. et

-12-

al., Cell 58:779-790 (1989)); the yeast recombinases
(Matsuzaki, H. et al., J. Bacteriol. 172:610-618 (1990));
the B. subtilis SpoIVC recombinase (Sato, T. et al., J.
Bacteriol. 172:1092-1098 (1990)); the Flp recombinase
5   (Schwartz, C.J. et al., J. Molec. Biol. 205:647-658
(1989); Parsons, R.L. et al., J. Biol. Chem. 265:4527-4533
(1990); Golic, K.G. et al., Cell 59:499-509 (1989); Amin,
A.A. et al., J. Molec. Biol. 214:55-72 (1990)); the Hin
recombinase (Glasgow, A.C. et al., J. Biol. Chem.
10 264:10072-10082 (1989)); immunoglobulin recombinases
(Malynn, B.A. et al., Cell 54:453-460 (1988)); and the Cin
recombinase (Hafter, P. et al., EMBO J. 7:3991-3996
(1988); Hubner, P. et al., J. Molec. Biol. 205:493-500
(1989)), all herein incorporated by reference.

15   Such systems are discussed by Echols, H. (J. Biol.
Chem. 265:14697-14700 (1990)), de Villartay, J.P. (Nature
335:170-174 (1988); Craig, N.L. (Ann. Rev. Genet.
22:77-105 (1988)), Poyart-Salmeron, C. et al. (EMBO J.
8:2425-2433 (1989)), Hunger-Bertling, K. et al. (Molec.
20 Cell. Biochem. 92:107-116 (1990)), and Cregg, J.M. (Molec.
Gen. Genet. 219:320-323 (1989)), all herein incorporated
by reference.

        Cre is the preferred recombinase of the present
invention because its site-specific recombinase activity
25 is dependent only upon the presence of the loxP site and
Cre. No energy is needed for this reaction; thus, there
is no requirement for ATP or other similar high energy
molecules. Moreover, no factors or proteins other than
the Cre protein is required in order to mediate sites
30 specific recombination at loxP sites (Abremski, K., et
al., J. Mol. Biol. Chem. 259:1509-1514 (1984); Hoess,
R.P., et al., Cold Spring Harbor Symp. Quant. Biol.
49:761-768 (1984), herein incorporated by reference).

        The use of plasmids having direct repeats of loxP
35 sit s t mediat gen cloning is discussed by Palazzolo,
M.J. et al. (Gene 88:25-36 (1990)), and Ell dge, S.J. et
al. (Proc. Natl. Acad. Sci. (U.S.A.) 88:1731-1735 (1991)).

-13-

Site-specific cleavage (but without a r combinas) is us d
in the "λZAP" gene cloning system of Stratagene (San
Diego, CA).

II. The Modulation of the Expression of the Preselected
5   Gene

     Because many preselected genes encode proteins whose
activities, like that of the Cre protein, are capable of
catalyzing forward and reverse reactions (under normal
host cell culturing conditions), it is desirable to be
10   able to modulate the expression of the preselected gene,
such that after a period sufficient to permit the
expression of the gene has transpired, further expression
will be limited, or will not occur.
     A nucleic acid molecule that encodes a recombinase,
15   such as Cre, may be used to recombine a target molecule
having loxP sites into a suitable loxP site-containing
vector. Similarly, it may be employed to remove a target
molecule from such a vector.

     A.   Reversible Modulation

20   In one embodiment of the present invention, the
nucleic acid molecule that contains the preselected gene
is constructed such that the expression of the preselected
gene can be reversibly modulated. As used herein, the
term "reversibly modulated" is intended to denote that the
25   expression of the gene can be repeatedly turned on and
off. Such modulation may be qualitative (i.e. in which
the preselected gene is either fully expressed, or fully
repressed) or quantitative (in which intermediate levels
of expression are possible). Most preferably, this
30   embodiment is achieved through the use of nucleic acid
molecul s that are vectors, and hence capable of
autonomous r plication within the host cell.

-14-

Suitable modulation can be obtained by expressing the preselected gene using inducible/de-repressible promoters and their respective inducers/de-repressors (for example, plac with IPTG, ptrp with IAA, λ pL with a λ temperature sensitive cI repressor, precA (or λ pL with a normal λ cI repressor) with UV light or mitomycin C, etc.).

Alternatively, the preselected gene can be mutated such that its product is conditionally active. Examples of such mutations include temperature sensitive mutations and "amber" mutations (when the host cell has a temperature sensitive amber suppressor), etc.

Where the preselected gene is naturally expressed on a bacteriophage, it is possible to employ the bacteriophage directly. Thus, for example, Cre can be provided to cells by infecting them with bacteriophage P1, the natural source of Cre. Desirably, a P1 mutant (such as a P1 which is deficient in replication, lysis, maturation, etc.) is employed to prevent cell death.

B.   Irreversible Modulation

In a preferred embodiment, the vector is constructed such that modulation of the preselected gene is irreversible. As used herein, the term "irreversible modulation" is intended to denote that once the expression of the gene has been turned off, it cannot be re-expressed. Thus, cessation of the expression of the preselected gene is permanent, and irreversible.

Such irreversible modulation is desirable when the preselected gene is a restriction endonuclease gene or a ligase. Irreversible modulation is particularly desirable in those cases wherein the preselected gene is capable of not only converting the target molecule into a desired r action product, but also conv rting the pr duced r action product back into the original target mol cule. Such modulation is theref re specially desirabl in the

-15-

case of a preselected gene that encodes a recombinase such
as Cre.

Irreversible modulation can be achieved in any of a
variety of ways. The preselected gene can be introduced
5   into a host cell as a non-vector molecule. Such a
molecule may, for example, be a linear or circular
molecule, a molecule lacking an origin of replication, or
a molecule that does not possess an origin of replication
that is recognized by the replicative apparatus of the
10   host cell, or a molecule that is not properly partitioned
upon cell division. When such a molecule is introduced
into the host cell, its preselected gene will be
expressed. However, due to the inability of the cell to
replicate the preselected gene, such expression will be
15   transient, and will ultimately cease as the cell divides
and loses the nucleic acid molecule that contains the
preselected gene. Since such molecules are incapable of
replication, they are ultimately degraded by nucleases or
other cellular processes.

20   In another embodiment, irreversible modulation is
achieved through the use of vectors (carrying the
preselected gene) that are conditionally incapable of
replication or partition in the host cell. Such vectors
may contain, for example, temperature sensitive replicons
25   or temperature sensitive partition proteins (such as parA
or parB of P1, sopA or sopB of P1, etc.). In this
embodiment, the host cell is incubated in the presence of
the target molecule and the vector under a replication
permissive temperature. After the desired expression of
30   the preselected gene has occurred, the temperature is
adjusted to a non-permissive temperature such that
additional replication of the vector is impaired.

In another embodiment, the vector that contains the
preselected gene may additionally contain and express a
35   "negative" selectable marker gene, such that the
expression of the marker gene imparts a c nditi nally
selectable disadvantage to th h st c 11.   In this

-16-

embodiment, the host cell is incubated in the presence of
the target molecule and the vector under permissive
conditions.   After the desired expression of the
preselected gene has occurred, the culturing conditions

5   are adjusted such that continued maintenance of the vector
is selected against.

         In a more preferred embodiment, the host cell will be
constructed such that it contains a "determinant" that has
the capacity to inhibit the expression of the preselected

10   gene.   As used herein, a "determinant" can be either a
site or regulatory element, or an expressible gene.   The
determinant may be present on the nucleic acid molecule
that contains the target molecule, on the nucleic acid
molecule that contains the preselected gene, or on any

15   other nucleic acid molecule (including the cell's
chromosome)).

         For example, the preselected gene may be present in
the vector in combination with recombinogenic or cleavable
sites, such that the expression of the preselected gene

20   results in the excision of the gene from the vector, and
the ultimate loss of the gene from the cell.   Most
preferably, the preselected gene and the recombinogenic
sites will be selected such that the excision reaction is
irreversible.   This can be accomplished, for example by

25   using the λint gene (encoding the Int recombinase) as the
preselected gene, and flanking this gene with an attP and
attB site.   Expression of Int will lead to the excision of
the λint gene from the vector, and the conversion of the
attP and attB sites into attL and attR sites.   Since Int

30   is effectively incapable of mediating recombination at
attL and attR sites (in the absence of the λ Xis protein),
it is unable to catalyze the reinsertion of the λint gene
into the vector.   Thus, the initial expression of the λint
gene r sults in an irreversible modulation   f its

35   expr ssion.

         In a similar manner, vectors c ntaining other
pr selected genes, such as the cre gene, can be employed.

For example, the host cell can be constructed to contain
and express the λ*int* gene (the gene may be present on the
target molecule, the nucleic acid molecule that contains
the preselected gene, or any other nucleic acid molecule
5   (including the cell's chromosome)), and the desired
preselected gene (for example, *cre*) can be present on a
nucleic acid molecule flanked by the *attP* and *attB* sites,
as described above. In lieu of the λ*int* gene, other
recombinases (such as mutant Cre proteins) or other
10   recombinogenic sites such as *loxP511* can be employed.

Most preferably, irreversible modulation of the
expression of the preselected gene will be accomplished
through the use of vector molecules having incompatible
replicons or incompatible partition requirements (Abeles,
15   A., *J. Biol. Chem.* *261*:3548-3555 (1986); Austin, S. *et
al.*, *Cell* *60*:351-354 (1990); Abeles, A. *et al.*, *Proc.
Natl. Acad. Sci. (U.S.A.)* *88*:9011-9015 (1991), all herein
incorporated by reference).

Preferably, incompatibility is mediated using the P1
20   replicon (which controls P1 copy number in cells). The P1
replicon consists of an origin sequence (P1 *ori*), an
essential replication protein (RepA) and dispensable
regulatory elements known as *incA* and *incC*. Naturally,
the *incA* element contains 9 copies of a DNA sequence
25   motif that binds RepA protein. Hence, by increasing the
number of RepA binding sites present in a cell, it is
possible to attenuate or inhibit the replication of P1 or
any vector that is replicated using the P1 replicon
(Abeles, A., *J. Biol. Chem.* *261*:3548-3555 (1986)).

30   Accordingly, the preselected gene is introduced into
the host cell on a vector that has a P1 *ori*, and a *repA*
gene. The vector may additionally contain one or more
incA and/or *incC* sites. More preferably, it will lack at
least ne, and most preferably all, f the *incA* sites
35   naturally associated with the P1 r plicon. Th abs nc of
th *incA* sites serves to enhance th copy number of the
vector (to about 10 copies per cell).

-18-

In this embodiment, the target molecule is adapted to
contain an incompatibility determinant comprising part or
all of the incA element (preferably comprising at least
two, and more preferably three RepA binding sites), such
that the introduction of the target molecule into a host
cell (containing the P1 ori vector) will prevent the
vector from replicating. Preferably, the target molecule
will be present in a suitable cloning site of a vector.
Desirably, this vector will have a replicon that results
in a high copy number per cell. The pUC replicon
(Yanisch-Perron, C. et al., Gene 33:103 (1985)) is
preferred for this purpose.

Cre protein (or nucleic acid encoding the Cre
protein) can also be provided to cells via the
introduction of capsules, liposomes, empty bacteriophage
particles, etc. Such introduction can be accomplished by
electroporation, micro-injection, infection, etc.

The present invention includes articles of
manufacture, such as "kits." Such kits will, typically,
be specially adapted to contain in close
compartmentalization a first container which contains a
first vector which contains, and is capable of expressing
a preselected gene, especially, the cre recombinase. The
first vector will additionally contain a replicon
(preferably a P1 replicon), such that the replication of
the first vector can be inhibited or prevented by the
presence of an incompatibility determinant. The
incompatibility determinant is contained on a second
vector molecule present in a second container of said kit.
The second vector molecule is adapted (as by possessing
restriction endonuclease cleavable cloning sites) to
receive a desired target nucleic acid molecule.
Preferably, the first vector molecule will lack at least
one, and preferably all f th incA associated RepA
binding sit s naturally present in the P1 replicon, and
will therefore have an enhanc d copy number (of about 10
c pies per c 11). Preferably, the s cond v ctor molecule

-19-

will be a high copy number plasmid (most preferably a
plasmid having a pUC replicon). The kit may additionally
contain instructional brochures, and the like. It may
also contain reagents sufficient to accomplish DNA cloning
5   (such as restriction endonucleases, ligases, buffers and
the like).

Having now generally described the invention, the
same will be more readily understood through reference to
the following examples which are provided by way of
10   illustration, and are not intended to be limiting of the
present invention, unless specified.

EXAMPLE 1

CONSTRUCTION OF A PREFERRED *CRE* EXPRESSION PLASMID: pZIP

A preferred Cre expression plasmid was made by
15   ligating together 3 PCR products each of which contained
a separate function. The three fragments are (1) the P1
ori, (2) the cre gene, and (3) a kanamycin resistance
determinant, Kan^R gene. The sources of these fragments
were as follows. Added restriction sites are underlined.
20   All P1 replicon coordinates are taken from Abeles, A. (J.
Biol. Chem. 261:3548-3555 (1986)); all cre gene
coordinates are taken from Sternberg, N. et al. (J. Molec.
Biol. 187:197-212 (1986)), both herein incorporated by
reference.

25   P1 ori:

To obtain a fragment that contains the P1 ori, PCR
amplification of the P1 origin was performed. For
such amplification, a left and right primer pair was
used. The left primer was about 70 bp upstream of
30   start of P1 ori (coordinate 291-309) and has a *Pst*I
site in its 5'-end (oligo #923)(SEQ ID NO:3):

5' CGAAGAGGTACTGCAGGGGCGATGAGCTTAAATGC 3'

The right primer was at very end of _repA_ gene
(coordinate 1501-1524) and has _Xba_I in 5'-end
(oligo #924)(SEQ ID NO:4):
#924: 5' GATCC<u>TCTAGA</u>TTATTCGGGGAGTTTCAGCTTTGG 3'
5   The primers were used with P1 _vir_ phage as
target, and the PCR yielded a 1261 bp product.

Cre gene:

The _cre_ gene of the vector was obtained through
PCR using a set of a left and a right primer
10   pair.   The right primer corresponded to
coordinate 1465-1484   + _Pst_I site (oligo
#793)(SEQ ID NO:5):
#793: 5' CGAAGAGGTA<u>CTGCAG</u>CAATCATTTACGCGTTAATGGC
3'

15   The left primer corresponded to coordinate
380-399 (oligo #925) + _Xba_I site (SEQ ID NO:6):
#925: 5' GATCC<u>TCTAGA</u>TAAAGGCAGAGCCGATCCTG 3'
The amplification yielded an 1134 bp product,
that included a single _cre_ promoter.
20   The primers were used against a P1/λ (P1/lambda)
hybrid phage (Sternberg, N., <u>J. Molec. Biol.</u>
<u>150</u>:603-609 (1981)).

Kan^R gene:

In order to obtain the kanamycin resistance
25   determinant, oligonucleotides which flank the
multiple cloning site of pUC4K (Pharmacia) were
used to give a PCR product of about 1400 bp with
flanking _Pst_I sites. The _Pst_I sites are not in
the primers - they are in the amplified sequence
30   in the multiple cloning site (MCS). The primers
that were used for this amplification are #790
(SEQ ID NO:7):
       5' CCATAACTTCGTATAATGTATGCTATACG-
          AAGTTATGGAAACAGCTATGACCATGAT 3'

-21-

and #791 (SEQ ID NO: 8):
                    5' CCATAACTTCGTATAGCATACATTATACG-
                    AAGTTATGTCACGACGTTGTAAAACGAC 3'

After PCR, reactions were digested with the following
restriction endonucleases:
    • P1 ori fragment: PstI and XbaI,
    • cre gene   : PstI and XbaI, and
    • kan^R gene   : PstI.
The resulting fragments were gel purified, mixed, and
ligated. The resulting product was transformed into E.
coli DH10B then selected for resistance to kanamycin
(Kan^R). The cre gene-containing plasmid was designated
pZIP. Colonies harboring "pZIP" showed Cre activity by in
vitro assay of crude cell sonicates.

                            EXAMPLE 2

                CONSTRUCTION OF A PREFERRED VECTOR FOR
                CONTAINING THE TARGET MOLECULE: λZipLox

        Vectors derived from bacteriophage λ often are used
for the construction of cDNA libraries, especially when
target clones will be identified by immunological
screening procedures (Huynh, T.V., In DNA Cloning: A
Practical Approach (Vol. 1) (D. M. Glover, ed.) IRL Press
Limited, Oxford, England, p. 49 (1985)). However, once a
clone is identified, the next step is usually to subclone
the cDNA into a plasmid vector to facilitate propagation,
characterization, and manipulation of the DNA.
        The use of the methods of the present invention
permit the automatic in vivo subcloning of DNA molecules
from λ vectors by exploiting the highly site-specific
Cre-loxP recombinational machinery of bacteriophage P1.
        λZipLox is the term used to designate the preferred
vector for containing the targ t molecul .

-22-

λZipLox is prepared by incorporating a plasmid, designated pZL, into a lambda phage. In the presence of Cre, the pZL plasmid is excised from the vector.

Plasmid pZL is a 4.3 kb derivative of GIBCO BRL
5 plasmid pSPORT1. Plasmid pZL was prepared by first using PCR to amplify that part of the incA region of the P1 regulon that containing repeats 1, 2, and 3. The PCR was performed against a P1 vir phage target using oligos #937 and #938 (coordinates 1939-1960 and 2043-2068,
10 respectively, as designated in Genbank record PP1REP, Genbank release 63.0) as primers:
(SEQ ID NO: 9)
#937: 5' GCGGCCAACATGGTGGGCACACATATTTGATACCAGCGA 3'
(SEQ ID NO: 10)
15 #938: 5' GGCCAAGATCTTGGACTGGTGAGAACGGCTTGCCCGGCAG 3'

This incA fragment was then cloned into the BspHI site (coord. 3569) of vector pSPORT1 (Life Technologies, Gaithersburg, MD) to make the construct pSAX10. When this region was sequenced, three mutations were found which
20 varied from the published sequence of incA: A instead of C at coordinate 1736, C instead of T at coordinate 1784, and A instead of G at coordinate 1819.

Next, a loxP site was cloned into the other BspHI site of the plasmid (coordinate 2561). This was
25 accomplished by PCR amplifying the Kanamycin resistance gene of plasmid pUC4K (Pharmacia) with primers #790 and #791. The resulting product contained the kan^R gene flanked by directly repeated lox sites. All other features of pSPORT1 are fully preserved in pZL.

30 This product was ligated to pSAX10 that had been cut with BspHI at coordinate 2561 and the ends made blunt. Kanamycin-resistant clones were then selected.

When the resulting plasmid was treated with Cre in vitro and transformed into E. coli cells, cl n s which had
35 become Kanamycin sensitive w re d tect d. On of th se was shown to have a functional loxP site and was designat d pZL.

-23-

λZipLox was constructed from ligation of a 23.1 kb
HindIII fragment derived from the left arm λgt10, a
12.7 kb SalI fragment derived from the right arm of a
deletion mutant of λgt11 [the 260 bp XhoI (position 33500)
to SalI (position 33240) fragment removed; lambda
coordinates are from Lambda II (Hendrix, R.W. et al.,
Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor,
New York (1983)) and pZL linearized by Cre-mediated
recombination with oligonucleotides to yield HindIII and
SalI compatible termini.

λZipLox is approximately 40.1 kb long, with a
predicted size limit for cDNA inserts of 10.1 kb based on
a theoretical capacity of 50.2 kb. Following the
conventions of Sambrook et. al. (Sambrook, J. et al., In:
Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold
Spring Harbor Laboratory, Cold Spring Harbor, New York
(1989)), the genotype of λZipLox is λsrIλ1[0] shndIIIλ1[0]
<loxP pMB1 ori lacI lacZ' T7 promoter-multiple cloning
site-SP6 promoter lacZ' incA amp loxP) sslIλ1[0] sxIλ1[0]
cIts857 srIλ4[0] nin5 srIλ5[0] Sam100. A simplified
representation of λZipLox is "left arm of bacteriophage
lambda - loxP site - plasmid pZL - loxP site - right arm
of bacteriophage lambda".

λZipLox contains seven unique restriction sites for
cloning cDNA within the multiple cloning site (EcoRI,
SalI, SstI, SpeI, NotI, XbaI and SplI). DNA inserts
cloned into the multiple cloning site of λZipLox reside
within the inducible lacZ' gene commonly found in pUC-type
plasmid vectors. When the lac promoter is induced with
IPTG, the cloned gene is expressed as a fusion protein
embedded within the amino-terminal portion of the
β-galactosidase fragment encoded by lacZ'. Thus, λZipLox
can be used to detect antigens expressed from cloned genes
in the same manner as the popular λgt11-based systems.

The λZipLox system solves th troublesome problem
associat d with the Cre-mediated recombinati n-d pend nt
automatic subcloning vector described by Palazz lo, M.J.,

-24-

et al. (Gene 88:25 (1990)). They reported reduced yields
of plasmids containing a LoxP site in cells expressing the
Cre recombinase constitutively.   The λZipLox system
eliminates this problem by incorporating a biological
5   switch into the λZipLox system that automatically reduces
the level of Cre recombinase after PZL is excised from
λZipLox: it uses the incompatibility between plasmids
containing the P1 origin of replication and the P1 incA
locus to mediate the elimination of the cre-producing
10   plasmid from the cell.

Immediately upon entering the cell, plasmid PZL (and
any DNA cloned into it) is excised and circularized from
the λZipLox genome by Cre-loxP recombination.   This
process also yields an intact λ phage, however, by simply
15   using a lambda lysogen as the host cell, the expression of
the λ phage can be repressed (by the resident λ prophage).
The preferred host for this purpose is a strain DH10BZIP.
DH10BZIP is a derivative of E. coli DH10B that was made by
lysogenization of DH10B (F- mcrA Δ(mrr hsdRMS-mcrBC)
20   φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697
araD139 galU galK nupG rpsL) with λNIH W82 (λxis6 ind-),
followed by transformation with pZIP. DH10BZIP expresses
Cre recombinase constitutively, and is thus preferred for
the automatic excision of PZL plasmids from clones
25   isolated from λZipLox libraries.   The introduction of
λZipLox (or any vector containing incA sites) will
irreversibly modulate Cre expression.

-25-

EXAMPLE 3
LAMBDA ZIPLOX PROTOCOL

A.   Ligation of CDNA to *Lambda* Ziplox

Lambda Ziplox *NotI-SalI* arms	0.5 μg	0.5 μg
CDNA *NotI-SalI* ends	50 ng	---
5X T4 DNA Ligase Buffer	1 μl	1 μl
T4 DNA Ligase (1 unit/μl)	1 μl	1 μl
autoclaved distilled water	to 5 μl	to 5 μl

1. Prepare two ligation mixes in two 1.5 ml autoclaved microcentrifuge tubes. One ligation mix does not have CDNA and will be the background.

2. Mix by pipetting up and down with pipettor. Minifuge briefly to collect contents at the bottom of the tube.

3. Incubate the ligation mixes at room temperature for 3 to 4 hours. Alternatively, the ligations can proceed overnight at 16°C.

B.   Packaging of the Ligation Mix

1. Use *Lambda* Packaging Mix (Life Technologies, Inc., Gaithersburg, MD, USA; cat. 8294SA), or equivalent, according to manufacture's directions.

2. Remove one blue tube (Extract A, Y00123, Life Technologies, Inc.) and one yellow tube (Extract B, Y01141, Life Technologies, Inc.) for each packaging reaction to be performed. Extract A is prepared from E. coli BHB2688 (lambda E⁻(am)); Extract B is prepared from E. coli BHB2690 (lambda D⁻(am)), see, Hohn, B. et al., Proc. Natl. Acad. Sci. (U.S.A.) 74:3259 (1977). Place both tubes immdiatly n ic .

-26-

One packaging reaction should be the Control *Lambda*
that comes with the packaging kit. It will serve as
a control to see if the packaging works. Use 2 µl
(0.5 µg) of the Control *Lambda*.

5   3. Thaw the extracts on ice. The contents should be
collected at the bottom of the tube by briefly
minifuging.

4. Transfer the entire ligation mix (5 µl) to the
blue tube as soon as the extracts have thawed.

10   5. Immediately transfer 15 µl from the yellow tube
into the blue tube and mix gently by stirring with
the pipette tip and gently pipetting up and down. Do
not vortex. Collect the contents at the bottom of
the tube by briefly minifuging.

15   6. Incubate at room temperature for 2 hours.

7. Add 0.5 ml phage dilution buffer to the tube. Add
20 µl of chloroform to the tube. Mix gently. Let
chloroform settle for 10 minutes, or minifuge for 1
minute. Store packaged phage at 4°C.

20   C.   Plating of Packaged *Lambda*

1. Grow up 5 ml of the appropriate strain (Y1090R⁻) in
TYN containing 10 mM Mg, 0.2% maltose, 0.1 mg/ml
ampicillin at 37°C. Use 0.5 A590/ml as the
concentration of cells to use to plate. They can be
25   diluted in phage dilution buffer.

2. Mix 100 µl of the diluted packaged phag with 200
µl of the diluted cells (Y1090R⁻) in a steril 13X100
glass tube or a sterile Falcon 2059 tub . Incubate
at 37°C for 15-20 minutes.

3. Add 3 ml molten (50°C) TYN top agar containing 10 mM Mg, 0.1 mg/ml ampicillin, and pour onto 100X15mm TYN plates.

4. When top agar is set (5 minutes), invert the plates and incubate overnight at 37°C.

5. The background packaged mix should be diluted to 1:100 for plating. The *Lambda* Ziplox with cDNA insert should be diluted to 1:100, 1:1000, and 1:10,000. The control *Lambda* should be diluted to 1:100,000 and 1:1,000,000.

D.   Excision

1. Grow DH10BZIP in TYN, 10 mM Mg, 0.2% maltose, 10 µg/ml kanamycin at 37°C.

2. Transfer a plaque from the plate to a sterile 1.5 ml microcentrifuge tube containing 250 µl of phage dilution buffer.

   a. With a scalpel or razor blade, cut about 5 mm off of a sterile pipette tip, so that the opening approximates the size of the plaque to be transferred.

   b. Place the shortened tip onto a P200 Pipetman and carefully aspirate the plaque into the tip.

   c. Transfer the plaque into the phage dilution buffer by pipetting up and down.

3. Incubat at room temperature 1-2 hours.

4. Mix 10 µl of the sup rnatant from th soaked plaque with 100 µl of DH10BZIP cells in a sterile

-28-

Falcon 2059 tube, and incubate at room temperature
for 15-20 minutes.

5. Add 400 µl SOC to each tube, and incubate shaking
at 37°C for 60 minutes.

5   6. Plate 5-20 µl onto a TYN plate containing
0.01% X-gal, 2 mM IPTG, and 0.1 mg/ml ampicillin.
This can be done by diluting the 5-20 µl into 100 µl
of SOC before spreading onto the plate.

7. Invert the plates and incubate them at 37°C
10   overnight.

The following reagents are used:

A.   Phage Dilution Buffer (50 mM Tris buffer pH 7.9, 100
mM NaCl, 10 mM MgCl, 0.01% gelatin. This solution
should be made up from sterile stocks and sterile
15   water, or it should be made up and then autoclaved.

B.   TYN media (10 gm Tryptone, 5 gm Yeast extract, 5 gm
NaCl, 10 ml 1 M Tris buffer pH 7.2, 225 µl 10 N NaOH,
water to 1 liter). Autoclave, then add the following
different reagents depending on what type of media is
20   required:
a. 10 mM Mg
Add 100 µl of 1M MgCl per 10 ml of media.
b. 0.1 mg/ml ampicillin
Add 100 µl of 10 mg/ml ampicillin per 10 ml of
25   media.
c. 10 µg/ml kanamycin
Add 10 µl of 10 mg/ml kanamycin to 10 ml of
m dia.
d. 0.2% malt se
30   Add 100 µl of 20% maltose t 10 ml of media.

C.   TYN plates (10 gm Tryptone, 5 gm Yeast extract, 5 gm
     NaCl, 10 ml 1M Tris buffer pH 7.2, 225 μl 10 N NaOH,
     17-20 gm agar.   Autoclave, and cool to about 60°C
     before adding other reagents. Then pour about 20 ml
5   into sterile petri dishes. Let set before storing
     them at 4°C. The following are different reagents
     that could be added depending on what type of plates
     are required.
     a. 10 mM Mg
10   Add 10 ml of sterile 1M MgCl to 1 liter of TYN.
     b. 0.1 mg/ml ampicillin
          Add 10 ml of sterile 10 mg/ml ampicillin to 1
          liter of TYN.
     c. 0.01% X-gal
15   Add 2.5 ml of 4% X-gal to 1 liter of TYN.
     d. 2 mM IPTG
          Add 20 ml of 100 mM IPTG to 1 liter of TYN.

D.   0.7% Top Agar (or agarose) (100 ml TYN media, and 0.7
     gm agar (or agarose).   Autoclave.   If using
20   immediately, place in a 60°C water bath to keep from
     solidifying. If it solidifies, re-melt it in a
     microwave oven. Be sure to loosen cap. Agarose is
     sometimes used instead of agar if plaque lifts are
     desired.   There is less chance of the agarose
25   sticking to the filters. If desired, the following
     may be added to the top agar:
     a. 10 mM Mg
          Add 10 μl of 1M MgCl per ml of top agar.
     b. 0.1 mg/ml ampicillin
30   Add 10 μl of 10 mg/ml ampicillin per ml of top
          agar.

E.   10 mg/ml ampicillin. Pr par d by w ighing ut 5 gm
     of ampicillin and adding it to 500 ml f distilled
     wat r. Mix until in soluti n. Sterile filt r the
35   solution and store at 4°C.

-30-

F. 1 M MgCl$_2$. Prepared by weighing out 20.33 gm of MgCl$_2$·6H20, and adding it to 100 ml of distilled water. Mix until in solution. Sterile filter or autoclave the solution.

5 G. 20% Maltose. Prepared by weighing out 20 gm of maltose and add it to enough distilled water to come to a final volume of 100 ml. Mix until in solution. Autoclave the solution.

H. 4% X-gal

10 1. Mix 1 gm of X-gal with enough dimethylformamide to come to a final volume of 25 ml. Store at -20°C.

I. 100 mM IPTG. Prepared by mixing 1 gm of IPTG with enough sterile distilled water to come to a final volume of 41 ml. Store at -20°C. Wrap container in 15 foil to keep light out.

J. 10 mg/ml kanamycin. Prepared by mixing 0.5 gm of kanamycin with enough sterile distilled water to come to a final volume of 50 ml. Store at 4°C. Wrap container in foil to keep light out.

20 In the above described examples, the following materials were obtained from Life Technologies, Inc.: T4 DNA Ligase and 5X T4 DNA Ligase Buffer (5224SB); *Lambda* Packaging System (8294SA); S.O.C. Medium (5544SA); X-gal (5520UC); and IPTG (5529UA).

-31-

EXAMPLE 4
CONSTRUCTION OF A cDNA LIBRARY IN λZIPLOX
AND EXPRESSION SCREENING

In order to verify the utility of the λZipLox system,
5   a HeLa cDNA library was constructed in λZipLox that had
been digested with Not I and Sal I.
E. Coli Y1090R⁻ [ΔlacU169 proA+ hadR- hsdM+ Δlon
araD139 strA supF[trpC22::Tn10] pMC9 (pMC9 = pBr322-lacI⁰)]
was cured of the resident plasmid, and the resulting
10   strain used to plate and screen the HeLa cDNA library. By
screening the induced library with primary antibody to
human vimentin, followed by reaction with alkaline
phosphatase-conjugated secondary antibody, four vimentin-
positive clones were recovered from approximately 10^5
15   plaques. The plasmids from these were excised in vivo,
and the 3'-terminal regions of the inserts sequenced. The
sequences of the four clones were identical to each other
(except for the length of the poly A tail derived from the
vimentic mRNA) over the 100 bp sequenced. However, the
20   sequence differed at six positions from the published
sequence for the 3' terminal region of human vimentin cDNA
(Ferrari, S. et al., Molec. Cell Biol. 6:3614 (1986)).
The excision process described here has several clear
advantages over other automatic subcloning techniques.
25   The excision efficiency (i.e., the conversion of input
phage particles to colonies) is about 50%. Moreover,
unlike M13-based systems, the λZipLox system does not
require infection with helper phage, and so is more rapid.
Selection for the desired excised recombinant is as simple
30   as infecting the Cre-expressing host cell with the λZipLox
clone of interest, and then selecting for Ap^R bacterial
colonies. Additionally, the vector does not pass through
a single-stranded intermediate that requires packaging
into a phage particle, pr cesses that may cause d 1 tions
35   in cloned ins rts (Michel, B. et al., Proc. Natl. Acad.
Sci. (U.S.A.) 84:3386 (1986)).

-32-

When plasmid DNA is prepared from clones after
excision, the distribution of plasmid between monomer and
dimer forms varies. This has practical consequences for
those who prefer to screen clones for plasmid insert size
5   by examining supercoiled DNA rather than linearizing or
excising the cloned fragment to estimate its size. For
the HeLa library, midscreen preparations from 17 randomly-
chosen clones showed that two yielded predominantly
monomer, while seven yielded predominantly dimer. The
10   remaining clones yielded an approximately equal amount of
each form of DNA. This may be a function of the effective
level of Cre recombinase at the time of infection, or an
indirect effect of the insert itself. The Cre recombinase
gene may be placed on a low copy-number replicon to
15   achieve the lowest level of Cre expression consistent with
efficient excision.

EXAMPLE 5

INCOMPATIBILITY BETWEEN pZL AND pZIP

In order to assess the effects of incompatibility
20   between pZL and pZIP, plasmids pSPORT (containing neither
loxP sites nor incA elements), pSPORTloxP (containing a
loxP site but no incA element), and pZL (containing, as
described above, both loxP sites and incA elements), were
introduced into the pZIP-containing E. coli strain
25   DH10BZIP. 100 μl of cells and 1 μl of plasmid were used
for each reaction. After the transformation, the cells
were plated on LB medium supplemented with ampicillin
(100 μg/ml) and methicillin (200 μg/ml). Five
transformants of each plasmid were selected and cultured
30   in SB medium supplemented with ampicillin (100 μg/ml) and
grown overnight.
Mini-preps were performed to assess yields of the
pSPORT-derived plasmids in DH10BZIP. 1 ml of the
v rnight culture was centrifuged, and plasmid was
35   extracted. The preparation was dissolved in 100 μl T$_{(1/10)}$E

-33-

buffer with RNase.   5 μl of each preparation was then
digested with ScaI in RE6 buffer, and the restriction
pattern was analyzed via electrophoresis (37°C, 30
minutes, 0.9% H-58).

5   The cells of the five colonies of pSPORT / pZip
control transformants were all found to contain both of
these plasmids.   The cells of the five colonies of
pSPORTLoxP / pZip control transformants were also found to
contain both plasmids, however, the yield of pSPORTLoxP
10   was much lower (estimated 5-10 fold) than pSPORT.   The
continued presence of the pZIP plasmid in these cells
indicates that, as expected, Cre expression had not been
modulated.

In contrast, the cells of the five colonies of pZL /
15   pZip transformants were all found to contain only the pZL
plasmid; the presence of the incA locus on the pZL plasmid
had successfully eliminated the pZIP plasmid. Moreover,
a substantial increase in pZL concentration was observed;
the yield of pZIP being higher than the yield of pSPORT.

20   These data indicate that the presence of Cre in
the cells (from the pZIP plasmid) had no effect on
replication of pSPORT (because pSPORT has no loxP site),
but severely inhibited replication of pSPORTLoxP
(presumably by binding to the loxP site and preventing
25   passage of the replication complex around the circular
molecule (see, Palazzolo, M.J., et al. (Gene 88:25
(1990)).   Incompatibility of pZL with pZip resulted in
rapid loss of pZip, and thus rapid loss of Cre protein,
allowing normal replication.   However, many of the pZL
30   molecules were present as dimers or higher multimers,
which may account for the higher yield of pZL than of
pSPORT.

Three transformant colonies of each of the above
transformati ns wer diluted 10³-fold in LB and then plated
35   n medium containing either ampicillin/m thicillin or
kanamycin. Th ability of cells to gr w in the presence
of ampicillin/methicillin indicates the pr senc of either

-34-

pSPORT, pSPORT_loxP_, or pZL. The ability of cells to grow
in the presence of kanamycin indicates the presence of
pZIP. The results of this experiment are shown in Table
1.

TABLE 1

TRANSFORMANT	COLONIES PER PLATE		SUMMARY DATA
	AMP	KAN	
pSPORT 1	913	1054	Σ_{amp} = 1,093
pSPORT 2	108	107	Σ_{kan} = 1,226
pSPORT 3	72	65	
pSPORT_LoxP_ 1	289	306	Σ_{amp} = 1,495
pSPORT_LoxP_ 2	871	936	Σ_{kan} = 1,565
pSPORT_LoxP_ 3	335	323	
pZL 1	185	0	Σ_{amp} = 922
pZL 2	307	0	Σ_{kan} = 0
pZL 3	430	0	

As shown in Table 1, all of the pZL transformants were
found to have lost the pZIP plasmid. When pZL /pZIP
transformants were grown on medium containing both
ampicillin and kanamycin no colonies formed, indicating
that dual selection could not "force" the maintenance of
the pZIP plasmid in the presence of pZL.

The experiment shown in Table 1 was repeated, except
that for the pZL / pZIP transformants, 1, 10, and 100 μl
of the undiluted stock of cells were plated. This
permitted a determination of whether the transformants
contained any kanamycin resistant cells. The results of
this experiment are shown in Table 2.

TABLE 2

TRANSFORMANT	COLONIES PER PLATE		SUMMARY DATA
	AMP	KAN	
pSPORT 1	140	138	Σ_{amp} = 544
pSPORT 2	183	191	Σ_{kan} = 496
pSPORT 3	221	167	
pSPORTLoxP 1	222	207	Σ_{amp} = 414
pSPORTLoxP 2	61	64	Σ_{kan} = 411
pSPORTLoxP 3	131	140	
pZL 1	98	0	Σ_{amp} = 979
pZL 2	167	0	Σ_{kan} = 0
pZL 3	714	0	

After adjustment for the dilution factor, the results indicated that the pSPORT transformants contained 1.8×10^6 amp^R cells / colony, and 1.65×10^6 kan^R cells / colony. Thus, the results indicate that all cells of the colony contain both pSPORT and pZIP. The results also indicated that the pSPORTLoxP transformants contained 1.38×10^6 amp^R cells / colony, and 1.37×10^6 kan^R cells / colony. Thus, the results indicate that all cells of these colonies contain both pSPORTLoxP and pZIP.

In contrast, although the pZL transformants contained 3.26×10^6 amp^R cells / colony, no kan^R cells were obtained. Thus, the pZL transformant colonies contained less than 10 kan^R cells / colony (i.e. less than 1 kan^R cell per 3×10^5 amp^R cells). The results indicate that the presence of the pZL plasmid led to the loss of the pZIP plasmid from the host cell.

In order to further demonstrate the utility of the present invention, the Cre-expressing host cell DH10BZIP was infected with λZipLox. After permitting phage adsorption, the cells were plated on medium containing ampicillin (100 µg/ml) and methicillin (200 µg/ml) and allowed to form colonies. The number of colonies which formed was approximat ly half that f th number of λZipLox phag s forming plaques, indicating that Cre-mediat d excision had occurred after infecti n in approximately 50% of input λZipLox. Col nies of

-36-

ampicillin/methicillin resistant cells were diluted and
plated on either medium containing ampicillin or medium
containing kanamycin, in the manner described above. The
ampicillin resistant colonies contained less than 1 kan^R
5   cell per 2 x 10^6 amp^R cells. The results indicate that the
presence of the pZL plasmid led to the efficient excision
of the pZIP plasmid from λZipLox, and that the presence of
the incA elements on pZL successfully rendered the host
cell incompatible to pZIP, and caused the loss of the pZIP
10   plasmid.

While the invention has been described in connection
with specific embodiments thereof, it will be understood
that it is capable of further modifications and this
application is intended to cover any variations, uses, or
15   adaptations of the invention following, in general, the
principles of the invention and including such departures
from the present disclosure as come within known or
customary practice within the art to which the invention
pertains and as may be applied to the essential features
20   hereinbefore set forth and as follows in the scope of the
appended claims.

-37-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: LIFE TECHNOLOGIES, INC.

    (ii) TITLE OF INVENTION: MODULATION OF ENZYME
          ACTIVITIES IN THE IN VIVO CLONING OF DNA

   (iii) NUMBER OF SEQUENCES: 8

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: WEIL, GOTSHAL & MANGES
        (B) STREET: 1615 L STREET, N.W.; SUITE 700
        (C) CITY: WASHINGTON
        (D) STATE: D.C.
        (E) COUNTRY: U.S.A.
        (F) ZIP: 20036

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Ver. #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: AUERBACH, JEFFREY I
        (B) REGISTRATION NUMBER: 32,680
        (C) REFERENCE/DOCKET NUMBER: 594-105-CIP

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (202) 682-7033
        (B) TELEFAX: (202) 859-0939

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

-38-

        (vi) ORIGINAL SOURCE:
              (A) ORGANISM: ESCHERICHIA COLI
              (B) STRAIN: DH10B

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5   ATAACTTCGT ATAATGTATG CTATACGAAG TTAT   34

    (2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 34 base pairs
              (B) TYPE: nucleic acid
10   (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

15   (vi) ORIGINAL SOURCE:
              (A) ORGANISM: ESCHERICHIA COLI
              (B) STRAIN: DH10B

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

    ATAACTTCGT ATAGCATACA TTATACGAAG TTAT   34

20 (2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 35 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (vi) ORIGINAL SOURCE:
30   (A) ORGANISM: ESCHERICHIA COLI
              (B) STRAIN: DH10B

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

    CGAAGAGGTA CTGCAGGGGC GATGAGCTTA AATGC   35

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (vi) ORIGINAL SOURCE:
        (A) ORGANISM: ESCHERICHIA COLI
        (B) STRAIN: DH10B

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCCTCTAG ATTATTCGGG GAGTTTCAGC TTTGG   35

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (vi) ORIGINAL SOURCE:
        (A) ORGANISM: ESCHERICHIA COLI

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGAAGAGGTA CTGCAGCAAT CATTTACGCG TTAATGGC   38

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

  (iii) HYPOTHETICAL: NO

-40-

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
            (A) ORGANISM: ESCHERICHIA COLI
            (B) STRAIN: DH10B

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCCTCTAG ATAAAGGCAG AGCCGATCCT G   31

      (2) INFORMATION FOR SEQ ID NO:7:

            (i) SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 57 base pairs
10   (B) TYPE: nucleic acid
                 (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear

           (ii) MOLECULE TYPE: DNA (genomic)

          (iii) HYPOTHETICAL: NO

15   (iv) ANTI-SENSE: NO

           (vi) ORIGINAL SOURCE:
                 (A) ORGANISM: ESCHERICHIA COLI
                 (B) STRAIN: DH10B

           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20 CCATAACTTC GTATAATGTA TGCTATACGA AGTTATGGAA ACAGCTATGA
CCATGAT   57

      (2) INFORMATION FOR SEQ ID NO:8:

            (i) SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 57 base pairs
25   (B) TYPE: nucleic acid
                 (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear

           (ii) MOLECULE TYPE: DNA (genomic)

          (iii) HYPOTHETICAL: NO

30   (iv) ANTI-SENSE: NO

           (vi) ORIGINAL SOURCE:
                 (A) ORGANISM: ESCHERICHIA COLI
                 (B) STRAIN: DH10B

           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

35 CCATAACTTC GTATAGCATA CATTATACGA AGTTATGTCA CGACGTTGTA
AAACGAC   57

-41-

WHAT IS CLAIMED IS:

1. A method for accomplishing in vivo alteration of
a target molecule in a host cell which comprises the
steps:
5   a) providing to said host cell a first vector, said
vector containing (i) a preselected gene and (ii) a
replicon, wherein said preselected gene is capable of
being expressed in said host cell, and said replicon is
sufficient to permit the replication of said vector;
10   b) providing to said host cell a second vector, said
vector containing (i) said target molecule which is to be
altered, (ii) a replicon, and (iii) a determinant that has
the capacity to inhibit the expression of said preselected
gene of said first vector;
15   c) culturing said host cell under conditions
sufficient to permit the expression of said preselected
gene, to thereby mediate the desired alteration of said
target molecule; wherein said second vector inhibits said
expression, and thereby results in the modulation of the
20   expression of said preselected gene.

2. The method of claim 1, wherein said inhibition is
reversibly modulatable.

3. The method of claim 1, wherein said inhibition is
irreversibly modulatable.

25   4. The method of claim 1, wherein said determinant
of said second vector inhibits the expression of said
preselected gene of said first vector by expressing a gene
present on said second vector.

5. The method of claim 4, wherein said expressed gene
30   of said second vector effects the excision of said
preselected gene from said first vector.

-42-

6. The method of claim 1, wherein said determinant of said second vector inhibits the expression of said preselected gene of said first vector by comprising an incompatibility determinant sufficient to inhibit the replication of said first vector, and thereby causing the loss of said vector from the said host cell.

7. The method of claim 6, wherein said replicon of said first vector is a P1 replicon, and wherein said incompatibility determinant comprises an incA regulatory element.

8. The method of claim 7, wherein said P1 replicon of said first vector lacks at least one naturally present incA regulatory element.

9. The method of claim 1, wherein said alteration is a recombinational alteration, and wherein said preselected gene is a recombinase gene, and said target molecule possesses at least one site recognized by said recombinase.

10. The method of claim 9, wherein said recombinase is Cre, and wherein said site is a loxP site.

11. The method of claim 6, wherein said alteration is a recombinational alteration, and wherein said preselected gene is a cre recombinase gene, and said target molecule possesses at least one loxP site.

12. The method of claim 7, wherein said alteration is a recombinational alteration, and wherein said preselected gene is a cre recombinase gene, and said target molecule poss sses at least one loxP site.

13. The m thod of claim 8, wher in said alteration is a recombinati nal alteration, and wherein said pres lect d

-43-

gene is a cre recombinase gene, and said target molecule
possesses at least one loxP site.

14. A host cell containing a first and a second
vector, wherein said first vector contains (i) a
preselected gene and (ii) a replicon, wherein said
preselected gene is capable of being expressed in said
host cell, and said replicon is sufficient to permit the
replication of said vector; and
wherein said second vector contains (i) a target molecule,
(ii) a replicon, and (iii) a determinant that has the
capacity to inhibit the expression of said preselected
gene of said first vector.

15. The host cell of claim 14, wherein said
determinant of said second vector inhibits the expression
of said preselected gene of said first vector by
comprising an incompatibility determinant sufficient to
inhibit the replication of said first vector.

16. The host cell of claim 15, wherein said replicon
of said first vector is a P1 replicon, and wherein said
incompatibility determinant comprises an incA regulatory
element.

17. The host cell of claim 16, wherein said P1
replicon of said first vector lacks at least one naturally
present incA regulatory elements.

18. The host cell of claim 15, wherein said
preselected gene is a cre recombinase gene, and said
target molecule possesses at least one loxP site.

19. The host cell of claim 16, wherin said
preselect d g ne is a cre rec mbinase gene, and said
target molecule possesses at least one loxP site.

-44-

20. The host cell of claim 17, wherein said preselected gene is a cre recombinase gene, and said target molecule possesses at least one loxP site.

21. A kit, being specially adapted to contain in close
5   compartmentalization:
    a) a first container which contains a first vector which contains a replicon and a preselected gene, said vector being capable of expressing said preselected gene in a host cell;
10 b) a second container, said container containing a second vector, said vector having a determinant that has the capacity to inhibit the expression of said preselected gene of said first vector, and being specially adapted to facilitate the introduction of a desired target molecule.

15 22. The kit of claim 21, wherein said determinant of said second vector inhibits the expression of said preselected gene of said first vector by comprising an incompatibility determinant sufficient to inhibit the replication of said first vector, and thereby causing the
20 loss of said vector from the said host cell.

23. The kit of claim 22, wherein said replicon of said first vector is a P1 replicon, and wherein said incompatibility determinant comprises an incA regulatory element.

25 24. The kit of claim 23, wherein said P1 replicon of said first vector lacks at least one naturally present incA regulatory elements.

25. The kit of claim 22, wherein said pr select d gene is a cr recombinase gene, and said target molecule
30 possesses at least ne loxP site.

-45-

26. The kit of claim 23, wherein said preselected gene
is a cre recombinase gene, and said target molecule
possesses at least one loxP site.

27. The kit of claim 24, wherein said preselected gene
5   is a cre recombinase gene, and said target molecule
possesses at least one loxP site.

International application No.

PCT/US93/00108

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 1/20; C12P 21/06; C07H 21/04
US CL :435/69.1, 172.3, 252.3, 321.1; 356/23.2, 24.1, 24.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 252.3, 321.1; 356/23.2, 24.1, 24.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, aps

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Mol. Biol., Vol. 192, issued 1986, Subrata K. Pal et al.: "P1 Plasmid Replication; Role of Initiator Titration in Copy Number Control", pages 275-285, see whole article.	1-27
A	Gene, Vol. 88, issued 1990, Michael J. Palazzolo et al., "Phage lambda cDNA cloning vectors for subtractive hybridization, fusion-protein synthesis and Cre-loxP automatic plasmid subcloning", pages 25-36, see whole article.	1-27
Y	Mol. Cellular Biol. Vol. 7 (6), issued June 1987, Brian Sauer, "Functional Expression of the cre-lox site-specific recombination system in the yeast Sacchararomyces cerevisiae", pages 2087-2096, see whole article.	1-27
A	PNAS (USA), Vol. 81, issued October 1984, Dhruba Chattoraj et al., "Plasmid P1 replication: Negative control by repeated DNA sequences", pages 6456-6460, see whole article.	1-27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 FEBRUARY 1993	31 MAR 1993

Name and mailing address f the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GARY L. BROWN
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Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US93/00108

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PNAS (USA), Vol. 88, issued October 1991, Ann L. Abeles et al., "Antiparallel plasmid-plasmid pairing may control P1 plasmid replication", pages 9011-9015, see whole article.	1-27